Diversity, ecological role and potential biotechnological applications of marine fungi associated to the seagrass Posidonia oceanica

Diversity, ecological role and potential biotechnological applications of marine fungi associated to the seagrass *Posidonia oceanica*

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Abstract

The marine environment is characterized by high salinity and exerts a strong selective pressure on the biota, favouring the development of halo-tolerant microorganisms. Part of this microbial diversity is made up of fungi, important organisms from ecological and biotechnological points of view. In this study, for the first time, the qualitative and quantitative composition of the mycoflora associated to leaves, rhizomes, roots and matte of the seagrass *Posidonia oceanica* was estimated. A total of 88 fungal taxa, mainly belonging to Ascomycota, were identified by morphological and molecular methods. The most represented genera were *Penicillium*, *Cladosporium* and *Acremonium*. Most of the species (70) were selectively associated with one district; only two species (*Penicillium chrysogenum* var. *chrysogenum* and *P. janczewskii*) were isolated from all the districts. Moreover the capability to produce laccases, peroxidases and tannases by 107 fungal isolated by the different districts of *P. oceanica* was carried out. These results show that the mycoflora associated to *P. oceanica* is very rich and characterized by fungi able to produce ligninolytic enzymes and tannases useful to degrade and detoxify lignocellulose residues in presence of high salt concentrations. These fungi, hence, may play important ecological roles in marine environments but can also be very useful in different biotechnological areas.

Highlights

► The mycoflora of *Posidonia oceanica* is very rich and district specific. ► Most of the isolated species are selectively associated with one district. ► Many of the screened fungi resulted laccase, peroxidase and tannase producers. ► Marine Ascomycetes attack the lignocellulose residues present in the sea. ► Marine Ascomycetes produce extremozymes active at high salt concentrations.

Introduction

Marine environments host a huge biodiversity of microorganisms, and fungi make up a large part of them: they show a high biodiversity, a specific habitat composition and seem to be important from both ecological and biotechnological points of view.

The basic knowledge on distribution and ecological role of marine fungi is still scarce. Marine fungi encompass a wide range of saprotrophs, parasites, symbionts, endophytes and epiphytes. They can be found in all marine habitats including planktonic communities, marine plants (i.e. algae, seagrasses, driftwood), marine invertebrates (sponges, coral, ascidians, bivalves, crustaceans, etc.), vertebrates (mainly fishes) and inorganic matter [1]. Recently, it has been estimated that marine fungi exceed 10,000 species/phylotypes despite many poorly investigated sources of marine fungi still exist [2]. To date, studies on marine-derived fungi have been conducted in deep sea [3] and [4], hypersaline environments [5], mangrove ecosystems [6], on decaying submerged woods [7], sponges [8] and corals [9], while other substrates have been poorly studied.

Fungi belonging to Ascomycota represent the predominant mycoflora in marine environments. Basidiomycota appear to be less present whereas, at the moment, an accurate estimate of other taxonomic groups in marine environments is impossible [4].

The functional significance of fungi in marine environments is still a subject of debate and analysis. In the past, their ecological importance was underestimated even if the different eco-physiological groups create communities that deeply affect the survival and productivity of marine ecosystems. They are involved in nutrient recycling, energy flow, and synthesis of humic-enzyme-exopolysaccharide complexes and mediate cycling between dissolved and particulate organic matter [3] and [10]. Hence, in addition to direct benefit or adverse effects, fungi can impact many environmental processes, particularly those associated with the decomposition of organic matter with special reference to lignocellulose residues [11]. For the few Basidiomycota present in the sea, extensive enzymatic degradation of all wood components was observed [12]. For marine Ascomycota, only few studies demonstrated their ability to degrade lignocellulose in the sea, thus further analysis must be carried out to understand this complex process [10].

The aim of this study was to isolate and identify the mycoflora associated with the different districts of the seagrass *Posidonia oceanica* and to study its capability to degrade this lignocellulose substrate very rich in tannins, assessing the capability to produce laccases, peroxidases and tannases, in presence of different salt concentrations.

Materials and methods

The study area was a *P. oceanica* meadow localized in Punta Manara close to Riva Trigoso Bay (Liguria, Italy). A total of nine plants with the surrounding matte (tangle of dead rhizomes and roots in which the sediment is trapped) were collected in March 2008 at a depth between −5 and −21 m. Plants were placed in sealed sterile bags and maintained at about 4°C during transport. Within few hours the samples were serially washed with sterile water and divided into four districts: leaves, rhizomes, roots and matte. Five grams (fresh weight) of each composite sample were homogenized in 100 ml seawater sterilized by filtration (0.2 μm pores). The homogenates were diluted 1:10 using sterilized seawater. The final dilutions of each composite sample were plated (1 ml per plate) on three media prepared with seawater with the addition of antibiotics (Gentalyn – 80 mg/l and Tazocin – 250 mg/l) to prevent bacterial growth: Glucose Peptone Yeast Agar (GPYA), Corn Meal Agar (CMA) and Agar Posidonia – AP (20 g of leaves, root, rhizomes and matte of *P. oceanica* in

seawater 100 ml heat up, 30 min to 60°C and filtrated; agar, 18 g; seawater make up the volume to one litre). Five replicates for each medium were prepared. Plates were incubated at 20°C (the average temperature of the Ligurian seagrass meadow in spring) and monitored daily for 30 days to allow development of slow-growing colonies. The number of CFU/g dw (colony forming units per g of dry weight of plant) was estimated. For each fungal morphotype in each district of *P. oceanica*, some strains were isolated in pure culture for the taxonomic identification.

Fungi were identified conventionally according to their macroscopic and microscopic features. After determination of their genera [13], [14] and [15], they were transferred to the media recommended by the authors of selected genus monographs for species identification. Fungal entities not identifiable morphologically and sterile mycelia (SM) were processed by molecular analysis by the sequencing of their ITS rDNA, their β-tubulin region (for *Penicillium* spp.) or their 26S rDNA (for yeasts), as previously described [16].

The nonparametric Mann–Whitney test for independent groups was run to assess the significance ($p \leq 0.05$) of the total fungal load differences (CFU/g dw) among the four *P. oceanica* districts on the same cultural medium, and between different media in the same district.

Principal Component Analysis (PCA) was used to visualize and evaluate qualitative and quantitative differences in the composition of the mycoflora of the four districts of *P. oceanica*. All statistics were obtained using for each species the highest average value found within the different media of each district of *P. oceanica*. PCA was performed using CANOCO [17] with focus scaling on inter species correlation while the species scores were divided by standard deviation. This last choice was taken to normalize the high values of *Penicillium brevicompactum* in the input matrix with respect to the other species. The centring and standardization by species were also applied and the results were shown using the biplot to visualize the species and the district in the same diagram [18].

Enzymatic screening

The enzymatic screening in presence of different salt concentrations (0, 15 and 30 g/l) was carried out on 107 strains isolated from *P. oceanica*. The ability to produce laccases and peroxidases was monitored through the ability to decolorize dyes with different redox potentials, Remazol Brilliant Blue (RBBR) and Amaranth Red (AmR), respectively [19]. Tannases production was monitored through the ability to degrade tannic acid (AcT) [20]. Fungal inocula consisted of mycelium disks (Ø 3 mm) inoculated in 48 well sterile polystyrene flat-bottom microtitre plates, containing 800 µl of the following media: RBBR 0 (glucose 20 g; malt extract 20 g; peptone 2 g; RBBR 0.2 g; H_2O 1l); RBBR 15 (as RBBR 0 + 15 g NaCl); RBBR 30 (as RBBR 0 + 30 g NaCl); AmR 0 (glucose 20 g; malt extract 20 g; peptone 2 g; AmR 0.2 g; H_2O 1l); AmR 15 (as AmR 0 + 15 g NaCl); AmR 30 (as AmR 0 + 30 g NaCl); AcT 0 (AcT 10 g; malt extract 20 g; peptone 2 g; H_2O 1l); AcT 15 (as AcT 0 + 15 g NaCl); AcT 30 (as AcT 0 + 30 g NaCl). Three replicates per medium were set up.

Plates were incubated in the dark at 24°C and sampled after seven days for the enzymes analyses. Samples of 200 µl per each well (diluted in water 2-fold, 4-fold and 500-fold for laccase, peroxidase and tannase, respectively) were transferred in sterile 96 flat-bottom wells polystyrene microtitre plates for spectrophotometric measurements. The degradation percentage (DP) was expressed as: $DP = 100\ (Abs_0 - Abs_7)/Abs_0$, where Abs_0 is the absorbance at time 0 and Abs_7 is the absorbance after seven days, measured at the maximum visible wavelength (λ_{max}) of RBBR, AmR and AcT. To evaluate the significance of differences ($p \leq 0.05$) in DP of RBBR, AmR and AcT at different salt concentrations, the multicomparison ANOVA test followed by the Bonferroni *post hoc* test was used.

Results

The total fungal load ranged from 1.4×10^2 to 1.6×10^3 CFU/g dw depending on the different *P. oceanica* districts and media used. Accordingly, the highest average value found for each taxa, within the different media of each district, was used for statistical analysis and comments of the results (Table 1).

Table 1.

Fungal load (CFU/g dw ± SD[c]) and number of taxa isolated from leaves, roots, rhizomes and matte of *P. oceanica* on three media (CMA, AP, GPYA [b])

	Leaves		Roots		Rhizomes		Matte		Total taxa[a]
	CFU/g dw ± SD	Number of taxa	CFU/g dw ± SD	Number of taxa	CFU/g dw ± SD	Number of taxa	CFU/g dw ± SD	Number of taxa	
CMA	$1.8 \times 10^2 \pm 8.1 \times 10^1$ aA	8	$2.0 \times 10^2 \pm 7.2 \times 10^1$ abA	7	$1.6 \times 10^3 \pm 2.6 \times 10^2$ aB	19	$1.4 \times 10^3 \pm 4.0 \times 10^2$ aBC	23	45 (27)
AP	$2.9 \times 10^2 \pm 1.2 \times 10^2$ abA	11	$4.8 \times 10^2 \pm 1.1 \times 10^2$ aAB	9	$8.8 \times 10^2 \pm 2.2 \times 10^2$ abBC	20	$1.1 \times 10^3 \pm 2.1 \times 10^2$ aC	22	43 (26)
GPYA	$5.2 \times 10^2 \pm 2.3 \times 10^2$ bAB	10	$1.4 \times 10^2 \pm 0.0$ bC	8	$4.4 \times 10^2 \pm 9.3 \times 10^1$ bB	11	$1.4 \times 10^3 \pm 3.2 \times 10^2$ aA	19	32 (13)
Total taxa	20		14		34		43		

Different uppercase indicate significant differences ($p \leq 0.0$, Mann–Whitney test) among the load of the same medium obtained in different district of *P. oceanica*; different lowercase indicate significant difference ($p \leq 0.0$ Mann–Whitney test) among the load of the same district of *P. oceanica* obtained in different media.

a

In brackets the number of exclusive taxa isolated from the specific medium.

b

CMA = Corn Meal Agar; AP = Agar Posidonia; GPYA = Glucose Peptone Yeast Agar.

c

CFU/g dw ± SD = colony forming units per g of dry weight of ±standard deviation.

Rhizomes displayed the highest fungal load, followed by matte, leaves and roots. A total of 88 taxa were identified: 43 from matte, 34 from rhizomes, 20 from leaves and 14 from roots (Table 1).

Most of the isolated species (70) were selectively associated with one district; only two species (*Penicillium chrysogenum* var. *chrysogenum* and *P. janczewskii*) were isolated from all the districts. This great dissimilarity in the mycoflora composition of the four districts of *P. oceanica* is well shown by PCA: matte is separated from the other districts along the first component axis, with high values of eigenvalues and percentage variance of species data. This is mainly due to the presence of 24 taxa exclusively associated to this district (Group 2) and to the high load of *P. brevicompactum* that displayed the highest eigenvalues (0.99) on axis 1 in the matrix of principal components (Fig. 1). Along the second component axis, rhizomes are separated from leaves and roots whose apparent closeness is mainly related to the almost or complete absence of species characterizing matte and rhizomes (i.e. *Phialophora bubakii*, *Stachylidium bicolor*, *Trichoderma harzianum* and *Penicillium spinulosum*). The separation along the second component axis is mainly due to the presence of groups of species exclusively isolated from rhizomes (22 taxa, Group 1), roots (8 taxa, Group 3) and leaves (15 taxa, Group 4), respectively and to the distribution of *P. janczewskii* that displayed the second highest eigenvalues (0.92) on axis 2 in the matrix of principal components (Fig. 1).

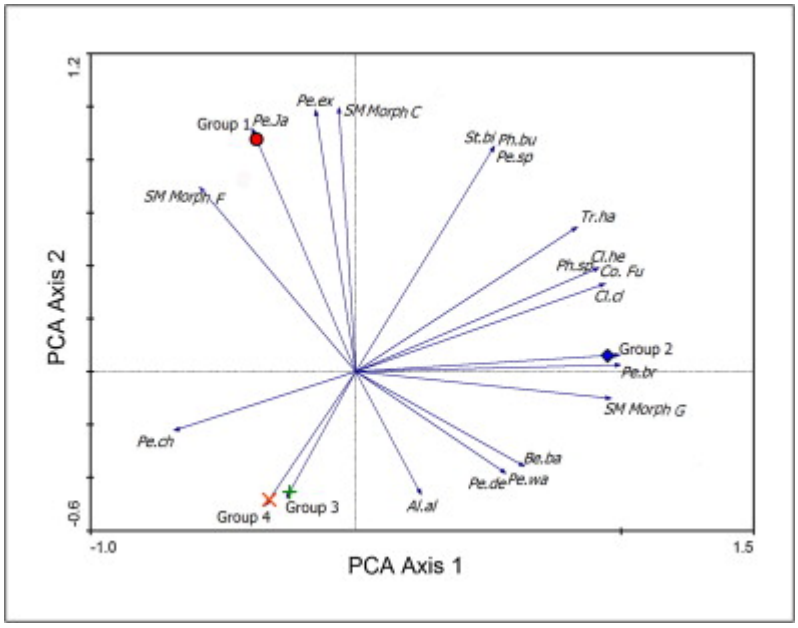

Figure 1.

Principal component analysis (PCA) of the mycofloras of the four districts of *P. oceanica* (for species name refer to Table 2). The first two axes are shown (eigenvalues: axis 1 = 0.827 and axis 2 = 0.138; cumulative percentage variance of species data: axis 1 = 82.7 and axis 2 = 96.6). ● = species exclusive of rhizomes (Group 1), ♦ = species exclusive of matte (Group 2), + = species exclusive of roots (Group 3), X = species exclusive of leaves (Group 4); *Al.al* = *A. alternata*; *Be.ba* = *B. bassiana*; *Cl.cl* = *C. cladosporioides*; *Cl.he* = *C. herbarum*; *Co.Fu* = *Coniothyrium fuckelii*; *Pe.br* = *P. brevicompactum*; *Pe.ch* = *P. chrysogenum* var. *chrysogenum*; *Pe.de* = *P. decumbens*; *Pe.ex* = *P. expansum*; *Pe.ja* = *P. janczewskii*; *Pe.sp* = *P. spinulosum*; *Pe.wa* = *P. waksmanii*; *Ph.bu* = *P. bubakii*; *Ph. sp.* = *Phaeosphaeria* sp.; *SM Morph.C* = *Pleosporales* sp. Sterile Mycelia morphotype C; *SM Morph. F* = *Pleosporales* sp. Sterile Mycelia morphotype F; *SM Morph.G* = Sterile Mycelia morphotype G; *St.bi* = *S. bicolor*; *Tr.ha* = *T. harzianum*.

On the whole, the 88 fungal entities comprised 78 Ascomycota and 4 Basidiomycota and 6 unidentified taxa (Table 2). A total of 41 genera were identified; the ones with the highest load and number of species in all districts were *Penicillium*, *Cladosporium* and *Acremonium*. The most abundant species were: *P. brevicompactum* and *Cladosporium cladosporioides* in matte; *P. janczewskii*, *Arthrinium sphaerospermum*, *Aspergillus fumigatus*, *P. chrysogenum* var. *chrysogenum* and *P. expansum* in rhizomes; *P. chrysogenum* var. *chrysogenum*, *Alternaria alternata*, *P. brevicompactum*, *P. janczewskii* and *Schizophyllum commune* in roots; *Penicillium waksmanii*, *P. chrysogenum* var. *chrysogenum* and *C. cucumerinum* in leaves.

Table 2.

Fungal entities isolated from leaves, rhizomes, roots and matte of P. oceanica, their mean load values (CFU/g dw[a]) and nucleotide sequences NCBI accession numbers.

Isolated taxa	Districts of *P. oceanica*				NCBI accession number
	Leaves CFU/g dw	Rhizomes CFU/g dw	Roots CFU/g dw	Matte CFU/g dw	
***Acremonium implicatum*(J.C. Gilman & E.V. Abbott) Gams 1975**	0	0	2.8×10^1	0	
***Acremonium minutisporum*(Sukapure & Thirum.) Gams 1971**	0	0	0	3.7×10^1	
***Acremonium*sp.**	0	7.3×10^1	0	0	
***Acremonium strictum*W. Gams 1971**	3.6×10^1	0	0	0	
***Acremonium tubakii*W. Gams 1971**	0	0	0	7.3×10^1	
***Acremonium verruculosum*W. Gams & Veenb.-Rijks 1971**	3.6×10^1	0	0	0	
***Alternaria alternata*(Fr.) Keissl. 1912**	0	0	5.7×10^1	3.7×10^1	KC339213
***Apiospora montagnei*Sacc. 1875**	0	0	0	3.7×10^1	
***Arthrinium phaeospermum*(Corda) M.B. Ellis 1965**	0	0	0	7.3×10^1	
***Arthrinium sacchari*(Speg.) M.B. Ellis 1965**[b]	0	0	0	3.7×10^1	KC339241
***Arthrinium sphaerospermum*Fuckel, 1873**	0	1.5×10^2	0	0	
Ascomycota sp. – SM morph. P[b]	0	7.3×10^1	0	0	KC339246
***Aspergillus awamori*Nakaz. 1915**	0	3.7×10^1	0	0	
***Aspergillus fumigatus*Fresen.,1863**	0	1.5×10^2	0	0	
***Aspergillus versicolor*(Vuill.) Tirab.,1908**[b]	3.6×10^1	0	0	0	KC339215

Isolated taxa	Districts of *P. oceanica*				NCBI accession number
	Leaves CFU/g dw	Rhizomes CFU/g dw	Roots CFU/g dw	Matte CFU/g dw	
***Beauveria bassiana*(Bals.-Criv.) Vuill., 1912**	0	0	2.8×10^{1}	3.7×10^{1}	
***Cadophora*sp. – SM morph. L**[b]	0	0	0	3.7×10^{1}	KC339236
***Candida zeylanoides*(Castell.) Langeron & Guerra 1938**	3.6×10^{1}	0	0	0	KC339243
***Cephalotrichum stemonitis*(Pers.) Nees 1809**	0	0	2.8×10^{1}	0	
***Cladosporium cladosporioides*(Fresen.) G.A. de Vries 1952**	0	1.1×10^{2}	0	2.9×10^{2}	
***Cladosporium cucumerinum*Ellis & Arthur 1889**	7.3×10^{1}	0	0	0	
***Cladosporium herbarum*(Pers.) Link 1816**	0	3.7×10^{1}	0	7.3×10^{1}	
***Cladosporium oxysporum*Berk. & M.A. Curtis 1868**	0	0	2.8×10^{1}	0	
***Cladosporium*sp.**	0	3.7×10^{1}	0	0	KC339216
***Cladosporium sphaerospermum*Penz. 1882**	0	7.3×10^{1}	0	0	
***Clonostachys rosea f. catenulata*(J.C. Gilman & E.V. Abbott) Schr.**	0	0	0	3.7×10^{1}	
***Coniothyrium fuckelii*Sacc. 1878**	0	3.7×10^{1}	0	7.3×10^{1}	KC339222
***Cremasteria cymatilis*Meyers & R.T. Moore (1960)**	3.6×10^{1}	0	0	0	
***Crocicreas cyathoideum*var.*cacaliae*(Bull)S.E. Carp.**[b]	0	3.7×10^{1}	0	0	KC339244
***Cyclothyrium*sp.**	0	0	0	3.7×10^{1}	KC339217
***Cylindrocarpon didymum*(Harting) Wollenw. 1924**	0	0	0	3.7×10^{1}	
***Dactylaria humicola*G.C. Bhatt & W.B. Kendr. 1968**	0	0	2.8×10^{1}	0	
***Diaporthe*sp.**[b]	0	3.7×10^{1}	0	0	KC339218
***Exophiala oligosperma*Calandron ex de Hoog & Tintel.,*et al.*(2003)**	0	0	0	3.7×10^{1}	KC339220

Isolated taxa	Districts of *P. oceanica*				NCBI accession number
	Leaves CFU/g dw	Rhizomes CFU/g dw	Roots CFU/g dw	Matte CFU/g dw	
***Geotrichum candidum*Link 1809**	0	0	0	3.7×10^{1}	
***Gibellulopsis nigrescens*(Pethybr.) Zare, W. Gams & Summerb. 2007**	0	0	0	3.7×10^{1}	KC339221
***Gliomastix murorum*var.*murorum*(Corda) S. Hughes 1958**	0	0	0	3.7×10^{1}	
Helotiales sp. – SM morph. I[b]	0	0	0	3.7×10^{1}	KC339237
***Massarina*sp.**[b]	0	7.3×10^{1}	0	0	KC339223
***Mycosphaerella punctiformis*(Pers.) Starback 1889**[b]	3.6×10^{1}	0	0	0	KC339234
***Myrmecridium schulzeri*var.*schulzeri*(Sacc.) Arza*et al.*, 2007**	3.6×10^{1}	0	0	0	
Myrothecium roridum Tode 1790	0	7.3×10^{1}	0	0	
Myrothecium verrucaria (Alb. & Schwein.) Ditmar 1813	0	3.7×10^{1}	0	0	
***Paraconiothyrium sporulosum*(W. Gams & Domsch) Ver.*et al.*, 2004**	0	1.1×10^{2}	0	0	KC339224
***Penicillium aurantiogriseum*var.*viridicatum*(Westling) Fris., 1989**	3.6×10^{1}	0	0	0	
***Penicillium brevicompactum*Dierckx,1901**	0	0	5.7×10^{1}	5.1×10^{2}	
***Penicillium chrysogenum*var.*chrysogenum*Thom, 1910**	1.1×10^{2}	1.5×10^{2}	1.7×10^{2}	3.7×10^{1}	KC339225
***Penicillium decumbens*Thom. 1910**	3.6×10^{1}	0	0	3.7×10^{1}	
***Penicillium expansum*Link 1809**	0	1.5×10^{2}	0	3.7×10^{1}	KC339226
***Penicillium italicum*Wehmer 1894**	0	1.1×10^{2}	0	0	
***Penicillium janczewskii*K.M. Zalessky 1927**	3.6×10^{1}	3.7×10^{2}	5.7×10^{1}	3.7×10^{1}	
***Penicillium restrictum*J.C. Gilman & E.V. Abbott 1927**	0	3.7×10^{1}	0	0	
***Penicillium spinulosum*Thom, 1910**	0	3.7×10^{1}	0	3.7×10^{1}	

Isolated taxa	Districts of *P. oceanica*				NCBI accession number
	Leaves CFU/g dw	Rhizomes CFU/g dw	Roots CFU/g dw	Matte CFU/g dw	
***Penicillium waksmanii*K.M. Zalessky, 1927**	1.5×10^{2}	0	0	7.3×10^{1}	
***Phaeosphaeria*sp.**[b]	0	3.7×10^{1}	0	3.7×10^{1}	KC339214
***Phialophora bubakii*(Laxa) Schol-Schwarz 1970**	0	3.7×10^{1}	0	3.7×10^{1}	
***Phialophora cinerescens*(Wollenw.) J.F.H. Beyma 1940**	3.6×10^{1}	0	0	0	
Pleosporales sp. – SM morph. E[b]	3.6×10^{1}	0	0	7.3×10^{1}	KC339245
Pleosporales sp. – SM morph. F[b]	0	0	2.8×10^{1}	7.3×10^{1}	KC339242
Pleosporales sp. – SM morph. M[b]	0	3.7×10^{1}	0	0	KC339239
Pleosporales sp. – SM morph. N[b]	0	0	0	3.7×10^{1}	KC339238
Pleosporales sp. – SM morph. O[b]	0	0	0	3.7×10^{1}	KC339248
Pleosporales sp. – SM morph. R[b]	0	0	0	3.7×10^{1}	KC339229
Pleosporales sp. – SM morph. A[b]	0	3.7×10^{1}	0	3.7×10^{1}	KC339240
Pleosporales sp. – SM morph. C[b]	0	3.7×10^{1}	0	0	KC339235
Pleosporales sp. – SM morph. Q[b]	0	0	0	3.7×10^{1}	KC339228
Pleosporales sp. – SM morph. T[b]	0	0	0	3.7×10^{1}	KC339219
***Pseudocercosporella fraxini*(Ellis & Kellerm.) U. Braun 1994**	3.6×10^{1}	0	0	0	KC339227
***Pycnidiophora dispersa*Clum 1956**	3.6×10^{1}	0	0	0	KC339231
***Pyrenochaeta cava*(Schulzer) Boerema, Loer. & Hamers 1996**	0	0	0	3.7×10^{1}	KC339230
***Pyrenochaeta*sp.**[b]	0	0	0	3.7×10^{1}	KC339232
***Radulidium epichloës*(Ellis & Dearn.) Arzanlou*et al.*, 2007**	0	0	0	3.7×10^{1}	
***Schizophyllum commune*Fr.1815**[b]	0	0	5.7×10^{1}	0	KC339233

Isolated taxa	Districts of *P. oceanica*				NCBI accession number
	Leaves CFU/g dw	Rhizomes CFU/g dw	Roots CFU/g dw	Matte CFU/g dw	
SM morph. B[b]	3.6×10^1	0	0	0	
SM morph. D[b]	0	0	2.8×10^1	0	
SM morph. G[b]	0	0	0	3.7×10^1	
SM morph. H[b]	0	0	0	3.7×10^1	
SM morph. J[b]	0	1.1×10^2	0	0	
SM morph. K[b]	0	0	2.8×10^1	0	
Sordariomycetes sp. – SM morph. S[b]	0	3.7×10^1	0	0	KC339247
***Sporobolomyces roseus*Kluyver & C.B. Niel 1924**	3.6×10^1	0	0	0	
***Stachylidium bicolor*Link 1809**	0	3.7×10^1	0	3.7×10^1	
***Torula herbarum*(Pers.) Link 1809**	0	0	2.8×10^1	0	
***Trichoderma harzianum*Rifai 1969**	0	3.7×10^1	0	3.7×10^1	
***Trichoderma koningii*Oudem. 1902**	0	3.7×10^1	0	0	
***Trichoderma*sp.**	0	3.7×10^1	0	0	
***Trichosporon lignicola*(Diddens) Fell & Scorzetti 2004**	0	3.7×10^1	0	0	
***Wallemia sebi*(Fr.) Arx 1970**	3.6×10^1	0	0	0	

a

CFU/g dw = colony forming units per g of dry weight of plant.

b

Sterile mycelia in axenic culture; SM morph. = sterile mycelium morphotype.

Twenty-nine morphotypes were SM in axenic culture. Molecular analysis showed that the majority of them belong to Dothideomycetes and in particular to the order Pleosporales. In several cases the analysis highlighted their proximity to unidentified endophytes isolated from both terrestrial and marine environments (mangrove plants and *Caulerpa racemosa*). For six morphotypes (SM morph. B, D, G, H, J and K) it was not possible to obtain a high quality DNA to amplify the specific sequences, despite the different attempts to extract it.

Enzymatic screening

RBBR decolorization

Fifty fungi were considered laccase producers because they showed, at least at one salt concentration, DP greater than 50% associated to low or no absorption. Among these, 21 displayed high DP (>75%) at least in one condition (Fig. 2).

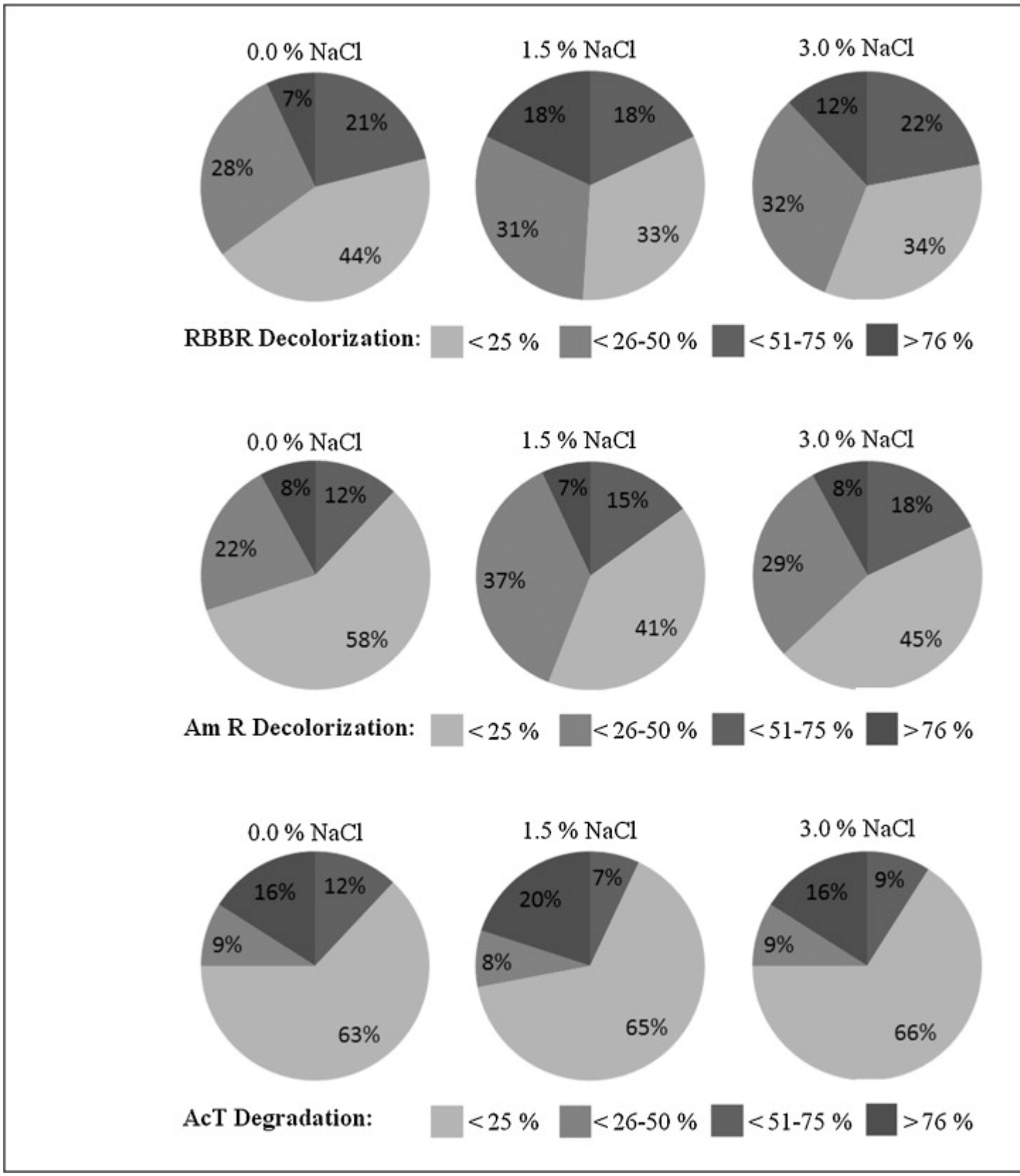

Figure 2.

Fungal capability to decolorize Remazol Brillant Blue (RBBR), Amaranth Red (AMR) and degrade tannic acid (AcT) in presence of different salt concentrations (0%, 1.5% and 3.0% (w/v) of NaCl): four groups were defined according to their efficiency (%).

Noteworthy strains were *C. cucumerinum* (MUT 4296) and SM morph. O (MUT 4399), which showed high DP at all salt concentrations. In addition *A. tubakii* (MUT 4268), *C. cladosporioides* (MUT 4292, 4295), *C. herbarum* (MUT 4300, 4301), *C. oxysporum* (MUT 4302) and *C. sphaerospermum* (MUT 4305) showed high DP at 15 and 30 g/l of salts whereas *B. bassiana* (MUT 4288) and *M. roridum* (MUT 4326) exclusively in absence of salt.

Amaranth red decolorization

Twenty-two fungi were considered peroxidase producers because they showed, at least at one salt concentration, DP higher than 50% associated to low or no absorption. Among these, 13 fungal strains showed high DP (>75%) at least in one condition (Fig. 2).

Noteworthy strains were *S. commune* (MUT 4387, 4388), which showed high DP at all salt concentrations. In addition *A tubakii* (MUT 4268), *C. cladosporioides* (MUT 4292) *C. oxysporum* (MUT 4302), *A. phaeospermum* (4278) and *C. herbarum* (MUT 4301) showed high DP at 15 and/or 30 g/l of salts, whereas *B. bassiana* (MUT 4288), *M. roridum* (MUT 4326) and *P. decumbens* (MUT 4346) exclusively without salt.

Tannic acid degradation

Twenty-nine fungi were considered tannase producers because they showed, at least at one salt concentration, DP higher than 50%. Among these, 21 strains showed high DP (>75%) at least in one condition (Fig. 2).

Noteworthy strains were *P. aurantiogriseum* var. *viridicatum* (MUT 4330), *P. brevicompactum* (MUT 4338), *P. chrysogenum* var. *chrysogenum* (MUT 4339, 4341, 4342), *P. decumbens* (MUT 4346), *P. expansum* (MUT 4348, 4349), *P. italicum* (MUT 4354), *P. janczewskii* (MUT 4357, 4358, 4360, 4362, 4363), *P. spinulosum* (MUT 4366, 4367) and *P. waksmanii* (MUT 4371), which showed high DP at all salt concentrations. Other strains belonging to *Penicillium*, *Cladosporium*, *Clonostachys*, *Aspergillus* and *Paraconiothyrium* displayed high DP in at least one condition.

Concerning the effect of salt concentration on enzyme activities, it must be underlined that most of the tested fungi were unaffected or even stimulated by the presence of high salt concentrations.

Discussion

The nature and diversity of filamentous fungi associated with marine organisms is still poorly understood. In this study, the qualitative and quantitative composition of the mycoflora associated to the seagrass *P. oceanica* was evaluated, with a focus on the fungal distribution in the different districts. Our results show that the mycoflora is very rich, both in terms of load and number of species, and is higher than that found on algae, corals, sponges and other seagrasses such as *Thalassia testudinum* and *Zostera marina* [8] and [21].

The use of different oligotrophic media prepared using seawater to mimic the natural environments and the incubation for prolonged time allowed to maximize the number of cultivable fungal entities and have certainly enhanced the chance of isolation of several slow growing, rare and less competitive species.

The mycoflora composition and structure change significantly in the four districts analysed, as shown by the PCA. This ‘district specificity’ may be due to multiple factors: specific environmental parameters (nutrients, light, exposure to hydrodynamic motions, etc.), presence of different

antagonistic microorganisms, particularly epiphytes or herbivores, presence of toxic or repellent molecules localized in a specific district of *P. oceanica*, that is, high concentrations of tannic acid in leaves and rhizomes [22]. The highest value of fungal load and species was found in rhizomes and matte, probably also in consequence to their high heterogeneity, which allows the formation of micro-habitats and niches rich in organic substances; these *P. oceanica* districts are capable of protecting fungi from predators and hydrodynamic motions and provide a great availability of nutrients.

Overall, by morphological and molecular analysis, 88 taxa mainly belonging to Ascomycota were identified, confirming that these fungi represent the predominant mycoflora in marine environments, where they play a central role in the organic matter decomposition and in different interactions with other organisms [21]. Fungi belonging to Basidiomycota and Mucoromycotina are instead less present in marine environments [23]. Among Basidiomycota, noteworthy is the recovery of *S. commune*, a white rot fungus already reported in mangrove ecosystem and known for its excellent ability to break down lignocellulose material [23].

The genera with the highest load and number of species were *Penicillium*, *Cladosporium* and *Acremonium*. Most species belonging to these genera and other abundant species observed in this analysis (*A. alternata*, *Aspergillus* spp., *Arthrinium* spp., *B. bassiana*, *Phialophora* spp. and *Trichoderma* spp.) are regarded as common inhabitants of marine environments because these are adapted to peculiar chemical and physical conditions. They perform important ecological functions, mainly in the decomposition of organic matter, in the recycling of elements and in the synthesis of humic compounds through the production of enzymes active in extreme conditions, often not produced by terrestrial analogues [11] and [12]. Moreover they represent a prolific source of biologically active secondary metabolites [24].

The few fungi (*Halotthia posidoniae*, *Pontoporeia biturbinata*, *Corollospora maritima*, *C. intermedia*, *Lulworthia* sp. and *Papulospora halina*) that were already reported by other authors as associated with *P. oceanica* [25] and [26] were not found in our survey. This result could be explained considering the season of the samplings: our study was conducted in spring on young plants, with fully green leaves apparently not colonized by epiphytes, while the other studies were conducted in autumn and winter on senescent plants displaying in all districts a typical pinkish-brown colour due to senescence and to the colonization by other organisms. Therefore, the differences found with respect to the other studies may be due to seasonality in the composition of the mycoflora of *P. oceanica* in relation to the life cycle of the plant or to the large epiphytic load that does not allow to evaluate whether the fungi are associated with the plant or with other organisms.

On the other hand, this study led to the recovery of species never or rarely reported from marine environments, such as *Acremonium implicatum*, *A. minutisporum*, *A. verruculosum*, *Aspergillus awamori*, *Cladosporium cucumerinum*, *C. oxysporum*, *C. tenuissimum*, *Cilindrocarpon didymum*, *Cephalotrichum stemonitis*, *Dactylaria humicola*, *Myrmecridium schulzeri* var. *schulzeri*, *Pycnidiophora dispersa*, *Pseudocercosporella fraxini*, *Radulidium epichloës*, *Schizophyllum commune* and *Trichosporon lignicola*. Some of them (*C. cucumerinum*, *D. humicola*, *M. schulzeri* var. *schulzeri* and *R. epichloës*) may have a pathosistic nutritional strategy, similar to their counterparts in terrestrial environments [27] and [28].

The enzyme screening clearly highlights that many marine fungi belonging to the phylum Ascomycota are capable of producing both the oxidative enzymes and the tannases useful to degrade lignocellulose residues. Approximately 50% of the tested fungi were able to decolorize extensively (DP > 50%) RBBR. This dye has been already used to highlight laccases activity [29]

because it has a redox potential similar to substrates attacked by this enzyme [29], [30] and [31]. A smaller but still high number of fungi (33%) were able to decolorize extensively AmR whose degradation is predictive of peroxidases production because of its higher redox potential similar to natural substrates of these enzymes [19] and [32].

Moreover many fungi (around 30%) were able to degrade AcT extensively. *P. oceanica* is characterized by high amounts of tannins ranging between 55 and 95 μg/g of dry weight of plant material according to the season and to phenomena of allelopathy against other seagrasses [33]. It is well known that tannins display antimicrobial and defensive properties. To the best of our knowledge, most of the fungi producing tannases are of terrestrial origin [34], with the only exception of a strain of *Aspergiullus awamori* isolated from seawater. This strain produces an acidophilic tannase that enables synthesis of propyl gallate by direct transesterification of tannic acid using propanol as organic reaction medium under low water concentration [35]. Our results, hence, underline for the first time the widespread ability to produce tannases even by marine fungi.

The high number of fungal strains able to transform RBBR, AmR and AcT strengthen the hypothesis that marine Ascomycota are able to attack *P. oceanica* residuals and other plant materials present in the sea, replacing the role of Basidiomycota in terrestrial environments, as already suggested by other authors [7]. The degradation of recalcitrant molecules, like lignin and tannins, by these fungi make available to other marine organisms cellulose or other simpler substances. Moreover, detoxifying the substrate, leaves and rhizomes of *P. oceanica* become available by other organisms from the early stages development. Thus, as decomposers of recalcitrant organic matter, marine Ascomycota play an important role in the cycle of the elements and allow large quantities of biomass to return in the food chain of the ecosystem.

As regards the influence of salt on enzymatic activities, about 2/3 of tested strains were not affected or even showed an increase of the degradative capacity with increasing salt concentration. This finding is extremely interesting because it states that these organisms are true marine fungi, active in extreme conditions (presence of high salt concentrations) that can trigger or increase the production of specific enzymes [7] and [36]. Actually, enzymes from marine organisms differ considerably from those produced by terrestrial counterparts because they are able to operate in extreme conditions (extremozymes) that generally would lead to protein denaturation [23]. These enzymes may be of great interest in future biotechnological applications where bioprocesses are carried out under extreme chemical–physical conditions [36] and [37].

In conclusion, the presented results contribute to the understanding of the ecological role of fungi in marine environments and in particular in *P. oceanica* meadows, a seriously threatened Mediterranean phytocenosis. The selection of strains with extremozymes with strong oxidative or tannase activity, even in the presence of high salt concentrations, opens new scenarios to their exploitation in different biotechnological areas.

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